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RELATED PCT APPLICATION NUMBER: *PCT/US05/01993*

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☑ *Additional inventors are being named on the* 1 *separately numbered sheets attached hereto*

TITLE OF THE INVENTION (500 characters max)

CYTOTOXICITY ASSAY

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ENCLOSED APPLICATION PARTS *(check all that apply)*

☑ Specification *Number of Pages* 8 ☐ CD(s), Number

☑ Drawing(s) *Number of Sheets* 16 ☐ Other (specify)

☐ Application Data Sheet. See 37 CFR 1.76

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Respectfully submitted,

SIGNATURE _____

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Date 01/23/2004

REGISTRATION NO. *(if appropriate)* 41,053

Docket Number: API-04-01-PR

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Docket Number	API-04-01-PR

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January 23, 2004
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PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

(DOCKET NO. API-04-01-PR)

TITLE: CYTOTOXICITY ASSAY

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CYTOTOXICITY ASSAY

Field of the Invention

The present invention relates to methods for detecting CTL activity using flow cytometry.

Background of the Invention

The ^{51}Cr-release assay has been the gold standard to measure CTL activity during an immune response for over 3 decades. However, the assay has a number of technical limitations and recent data has brought into question the physiological relevance of the ^{51}Cr-release assay and whether it is a true measure of CTL activity measured against target cells *in vivo*, especially in solid tumors. The lysis of target cells in *in vitro* CTL assays can be induced via two disctinct mechanisms: 1) A membranolysis process mediated when perforin is simply inserted into the target cell membrane; and, 2) DNA fragmentation (apoptosis) induced by the action of Granzyme B and Granzyme A entering a target cell via perforin and/or vesicle fusion events at the target cell surface. The ^{51}Cr-release assay measures the first of these two read-outs. The latter mechanism has more physiologic relevance and is required *in vivo* for target cell killing. Recent observations indicate that measurement of effector cell cytotoxicity by the ^{51}Cr-release assay may not be accurate or in some cases, even relevant. Based on these observations, the investigator must remain cognizant of the fact that effector cells determined to be cytotoxic based on ^{51}Cr-release assays may indeed not be CTL capable of tumor cell killing via DNA fragmentation *in vivo*. This may account for many of the results in the cancer immunotherapy literature in which effector cell responses monitored *in vitro* may not correlate with vaccine efficacy in the patient. For example, peptide vaccines selected by virtue of positive effector cell cytotoxicity date generated using ^{51}Cr-release assays may not accurately reflect the actual epitopes active in inducing CTL lysis *in* vivo via DNA fragmentation. In addition, there are a number of other issues plaguing the use of ^{51}Cr-based assays, including the need to use hazardous radioactivity, and problems related to the use of scintillation counters.

The JAM assay is a newer method that solves some of the issues with ^{51}Cr-release. Although it measures DNA fragmentation effectively, it has serious limitations due to the need to label the DNA of target cells with agents such as ^{3}H-thymidine or ^{125}I-deoxyuridine. This method invariably causes DNA damage and changes the physiology of the target cell before the CTL assay. In addition, the ^{3}H-thymidine labelling method does not afford the same degree of sensitivity as ^{51}Cr and ^{125}I is a dangerous agent requiring considerable lead shielding of both the lab worker as well as the lab area. Overall, a non-radioactive method to measure apoptosis induction and DNA fragmentation in target cells during CTL attack would be a more ideal assay, especially when contemplating its use in clinical trial monitoring.

Detection of capsase cleavage using fluorogenic caspase substrates has also been used to demonstrate cytotoxic T cell activity (Liu, et al. Visualization and quantification of T cell-mediated cytotoxicity using cell-permeable fluorogenic caspase substrates. Nautre Medicine, Jan. 2003, 9(1):4-5.) In addition, other highly sensitive methods to enumerate antigen-specific $CD8^{+}$ T-cells have been introduced, such as intracellular cytokine staining, the ELISPOT assay, and staining for T-cells using class I MHC tetramers. However, these assays do not measure ultimate cytolytic function, which is especially important in analyzing anti-tumor immune responses. Assays offering a suitable levels of sensitivity, specificity, safety and ease-of-use are lacking. A new and sensitive assay system for detecting CTL activity is provided herein.

Summary of the Invention

The present invention provides a cytotoxicity assay that detects DNA fragmentation- and/or apoptosis-related changes in target cells following contact with effector cells. In one embodiment, the assay is conducted using one or more reagents that detect cleavage of a caspase or phosphorylation of histones. In a preferred embodiment, cleavage of caspase is detected using a monoclonal antibody. In another preferred embodiment, phosphorylation of histones is detected using a monoclonal antibody. In other embodiments, cleaved caspase and/or phosphorylated histones are detected using at least one monoclonal antibody coupled with flow cytometric analysis.

Brief Description of the Drawings

Figure 1. Assay protocol.

Figure 2. Flow cytometric analysis of data.

Figure 3. Assay conducted using P815 target cells.

Figure 4. Mouse MLR CTL activity.

Figure 5. Antigen-Specific Mouse CTL Assay.

Figure 6. Assessment of the immune response to TRP2 peptide linked to hPER1 PTD sequence.

Figure 7. Peptide-Specific Mouse CTL Assay.

Figure 8. Human T Cell Activation Assay.

Figure 9. Human CTL Assay Results.

Figure 10. Dilution of Effectors with Naïve Cells.

Figure 11. Dilution of Effectors with Naïve Splenocytes.

Figure 12. CTL Activity in Mouse MLR: Diluted Effects.

Figure 13. Sensitivity of Caspase-3 Assay in Effector Dilution.

Figure 14. Flow cytometric analysis of a two-hour target/effector incubation.

Figure 15. Flow cytometric analysis of a four-hour target/effector incubation.

Figure 15. Flow cytometric analysis of a six-hour target/effector incubation.

Detailed Description

The present invention relates to reagents and assays for performing cytotoxicity assays. The assays are suitable to detecting cytotoxicity mediated by immune effector cells including but not limited to T cells, natural killer (NK) cells, granulocytes, monocytes, macrophages, and the like. The assays are generally based on the detection of DNA fragmentation- and/or apoptosis-related changes in a target cell. For instance, in certain embodiments, cleavage of an enzyme involved in the apoptotic process is detected. In another, a change in the characteristic of a DNA-associated protein during the apoptotic process is detected. Such changes are suitable to detection using devices that measure cell fluorescence, such as a Fluorescence Activated Cell Sorter (FACS) machine or flow cytometer.

In one embodiment, the present invention relates to an assay for measuring the cleavage of one or more caspases during the apoptotic process. Caspases shown to play a role in apoptosis that may be suitable for detection in an assay as shown herein many include but are not limited to caspase 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, and 14. In one embodiment, caspase 3 is detected using the assay described herein. Another enzyme known to be cleaved during the apoptotic process is poly-ADP ribose polymerase (PARP). Cleavage of caspases, in particular caspase 3, and PARP are early events during apoptosis and are triggered in CTL targets. In addition to the cleavage of caspases and PARP, a number of intracellular substrates are phosphorylated during the induction of apoptosis. For instance, histone H2A.X is phosphorylated in wide variety of cell lineages. Antibodies with specific binding capacity for cleaved caspases, as well as antibodies specific for phosphorylated histone H2.AX are commercially available and suitable for use in practicing the present invention.

In certain embodiments, dyes are utilized to stain the target cells prior to contact with effector cells. Suitable dues include Vial T, CFSE, and DDAO-SE, each of which are known in the art. A preferred dye is DDAO-SE (Molecular Probes). Other suitable dyes are also known in the art and may be useful in practicing the present invention.

In one embodiment, the assay of the present invention provides is carried out by: 1) preparing effector cells (E); 2) preparing target cells (T), including tagging the cells with a detectable marker such as a fluorescent dye; 3) mixing the effector cells and the target cells at a sufficient number of E:T ratios; 4) incubating the mixture for a sufficient period of time and under conditions suitable for at least some of the effector cells to become cytotoxic toward at least some of the target cells; 5) fixing and permeabilizing the mixed cells; 6) staining the cells with a detectable reagent having the ability to bind a protein or fragment thereof that undergoes a change during or results from the apoptotic process; and, 7) detecting the detectable reagent. In certain embodiments, the detectable reagent is detectable by virtue of a fluorescent tag or moiety contained within the reagent or by detecting a secondary reagent that binds to the detectable reagent, the secondary reagent being detectable by virtue of a fluorescent tag or moiety contained within the secondary reagent. In preferred embodiments, the detectable reagent and / or the secondary reagent is an antibody, which may be fluorescently labeled. The detectable

reagent may detected by any of several well-known methods including but not limited to FACS. In one preferred embodiment, the detectable reagent is an anti-Caspase 3 antibody (such as the PE Conjugated Monoclonal Rabbit Anti-Active Caspase 3 Antibody, BD Pharmingen Catalog No. 550914). Other suitable reagents are known in the art.

In certain embodiments, it may be beneficial or desirable to contact or "pulse" a target cell with a peptide to which the effector cells may respond prior to staining the target cells and / or mixing the target and effector cells. For instance, a target cell may be pulsed with a peptide corresponding to the amino acid sequence of an infectious agent such as HIV or a tumor antigen. Suitable tumor antigens include include, for example, gp100 (Cox et al., *Science*, 264:716-719 (1994)), MART-1/Melan A (Kawakami et al., *J. Exp. Med.*, 180:347-352 (1994)), gp75 (TRP-1) (Wang et al., *J. Exp. Med.*, 186:1131-1140 (1996)), tyrosinase (Wolfel et al., *Eur. J. Immunol.*, 24:759-764 (1994)), NY-ESO-1 (WO 98/14464; WO 99/18206), melanoma proteoglycan (Hellstrom et al., *J. Immunol.*, 130:1467-1472 (1983)), MAGE family antigens (i.e., MAGE-1, 2,3,4,6, and 12; Van der Bruggen et al., *Science*, 254:1643-1647 (1991); U.S. Pat. Nos. 6,235,525), BAGE family antigens (Boel et al., *Immunity*, 2:167-175 (1995)), GAGE family antigens (i.e., GAGE-1,2; Van den Eynde et al., *J. Exp. Med.*, 182:689-698 (1995); U.S. Pat. No. 6,013,765), RAGE family antigens (i.e., RAGE-1; Gaugler et at., *Immunogenetics*, 44:323-330 (1996); U.S. Pat. No. 5,939,526), N-acetylglucosaminyltransferase-V (Guilloux et at., *J. Exp. Med.*, 183:1173-1183 (1996)), p15 (Robbins et al., *J. Immunol.* 154:5944-5950 (1995)), ß-catenin (Robbins et al., *J. Exp. Med.*, 183:1185-1192 (1996)), MUM-1 (Coulie et al., *Proc. Natl. Acad. Sci.* USA, 92:7976-7980 (1995)), cyclin dependent kinase-4 (CDK4) (Wolfel et al., *Science*, 269:1281-1284 (1995)), p21-*ras* (Fossum et at., *Int. J. Cancer*, 56:40-45 (1994)), BCR-*abl* (Bocchia et al., *Blood*, 85:2680-2684 (1995)), p53 (Theobald et al., *Proc. Natl. Acad. Sci.* USA, 92:11993-11997 (1995)), p185 HER2/neu (erb-B1; Fisk et al., *J. Exp. Med.*, 181:2109-2117 (1995)), epidermal growth factor receptor (EGFR) (Harris et al., Breast Cancer Res. Treat, 29:1-2 (1994)), carcinoembryonic antigens (CEA) (Kwong et al., *J. Natl. Cancer Inst.*, 85:982-990 (1995) U.S. Pat. Nos. 5,756,103; 5,274,087; 5,571,710; 6,071,716; 5,698,530; 6,045,802; EP 263933; EP 346710; and, EP 784483); carcinoma-associated mutated mucins (i.e.,

MUC-1 gene products; Jerome et al., *J. Immunol.*, 151:1654-1662 (1993)); EBNA gene products of EBV (i.e., EBNA-1; Rickinson et al., *Cancer Surveys*, 13:53-80 (1992)); E7, E6 proteins of human papillomavirus (Ressing et al., *J. Immunol*, 154:5934-5943 (1995)); prostate specific antigen (PSA; Xue et al., *The Prostate*, 30:73-78 (1997)); prostate specific membrane antigen (PSMA; Israeli, et al., *Cancer Res.*, 54:1807-1811 (1994)); idiotypic epitopes or antigens, for example, immunoglobulin idiotypes or T cell receptor idiotypes (Chen et al., *J. Immunol.*, 153:4775-4787 (1994)); KSA (U.S. Patent No. 5,348,887), kinesin 2 (Dietz, et al. Biochem Biophys Res Commun 2000 Sep 7;275(3):731-8), HIP-55, TGFβ-1 anti-apoptotic factor (Toomey, et al. Br J Biomed Sci 2001;58(3):177-83), tumor protein D52 (Bryne J.A., et al., Genomics, 35:523-532 (1996)), H1FT, NY-BR-1 (WO 01/47959), NY-BR-62, NY-BR-75, NY-BR-85, NY-BR-87 and NY-BR-96 (Scanlan, M. Serologic and Bioinformatic Approaches to the Identification of Human Tumor Antigens, in *Cancer Vaccines 2000*, Cancer Research Institute, New York, NY), among others, including wild-type, modified, mutated versions thereof.

In certain embodiments, the time during which the target and effector cells are incubated may be modified. For instance, it may be desirable to incubate the mixture for one, two, three, four, five, six or more hours prior to further analysis.

Other aspects of the present invention include kits for carrying out the assays described herein. A kit may include materials useful in preparing effector cells, a suitable number of effector cells, a fluorescent dye with which to stain the target cells, a device such as a 96-well plate in which the target and effector cells may be mixed and incubated, materials required for fixing and permeabilizing the cells, a detectable reagent, and / or a secondary reagent. Different combinations of such materials may be organized as a kit in order to aid the skilled artisan in carrying out the assay of the present invention.

A better understanding of the present invention and of its many advantages will be had from the following examples, given by way of illustration.

EXAMPLES

A sensitive flow cytometry-based assay for the measurement of antigen-specific CTL function based on the activation of caspase 3 and/or the phosphorylation of histones

(histone H2A.X) in target cells induced to undergo apoptosis by CTL through the action of perforin and granzymes is described below. As outlined in **Figs. 1, 5, and 8**, target cells were labeled with the cell membrane tracking dye called DDAO-SE (Molecular Probes), incubated with CTL, fixed, permeabilized, and stained with with an antibody recognizing cleaved caspase 3 and/or phosphorylated histone H2A.X. The cells were then assayed by flow cytometry. The assay was tested in a number of different human and mouse systems generating antigen-specific $CD8^+$ T-cells, including allogeneic responses and peptide-specific T-cell responses induced *in vitro*, or after vaccination in mice (**Figs. 2-4, 6, 7, and 9**). The assay was found to be as sensitive as MHC tetramer-based methods with detection of antigen-specific CTL activity at frequencies on the order of 0.25 to 0.1% (1:400 to 1:1,200) (**Figs. 10-16**). When murine peptide-specific CTL responses were determined *ex* vivo in cell suspensions from peptide-immunized mice, the new assay exhibited sensitivities equal to or greater than ^{51}Cr-release with lower backgrounds. These results suggest that this flow cytometry-based assay is a powerful tool to determine CTL responses in clinical trial monitoring as well as pre-clinical experiments of CTL function. The assay also has the advantage of also tracking the fate of the CTL effector cells and can be improved by staining for additional products of apoptosis in target cells.

While the present invention has been described in terms of the preferred embodiments, it is understood that variations and modifications will occur to those skilled in the art. Therefore, it is intended that the appended claims cover all such equivalent variations that come within the scope of the invention as claimed.

FIGURE 1
Assay Protocol

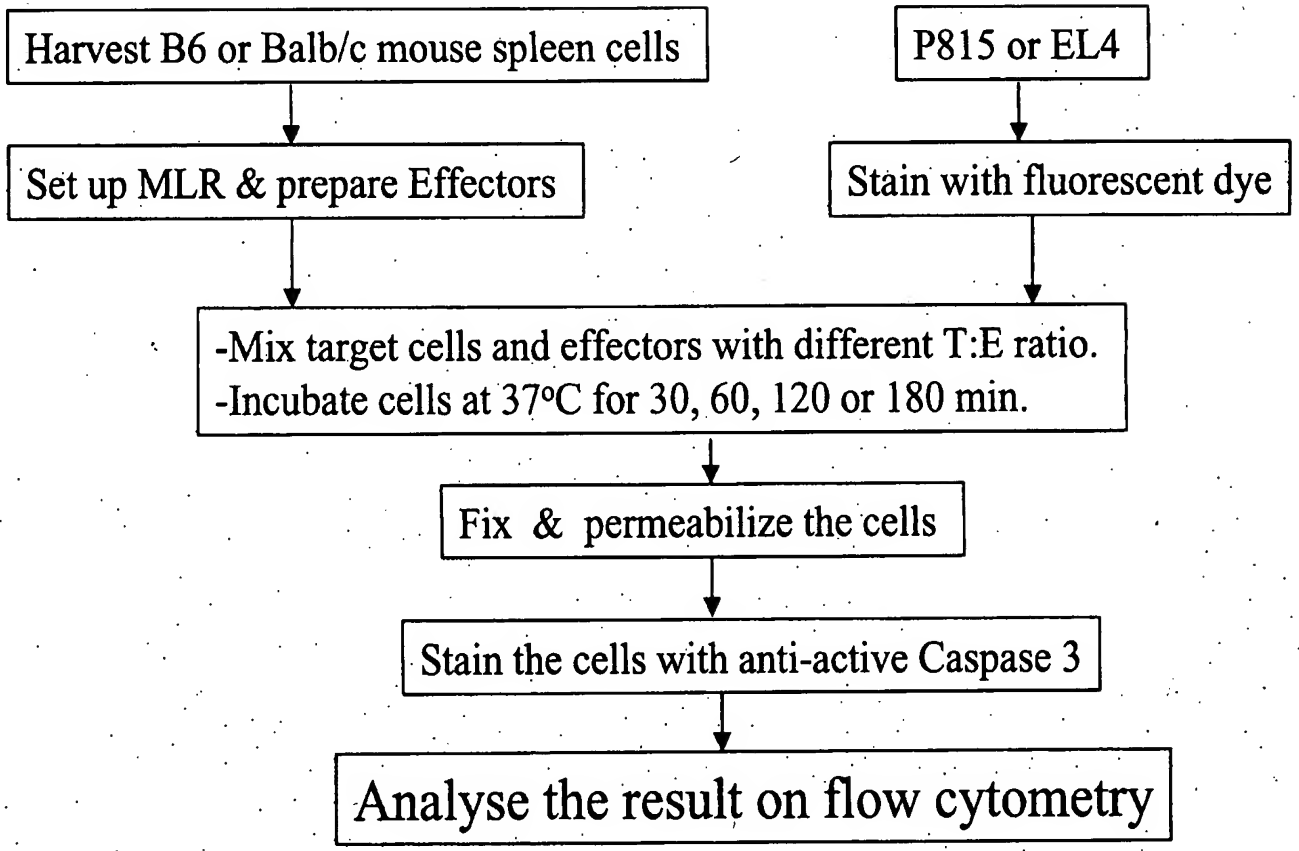

FIGURE 2
Flow Cytometry Data Analysing Methods

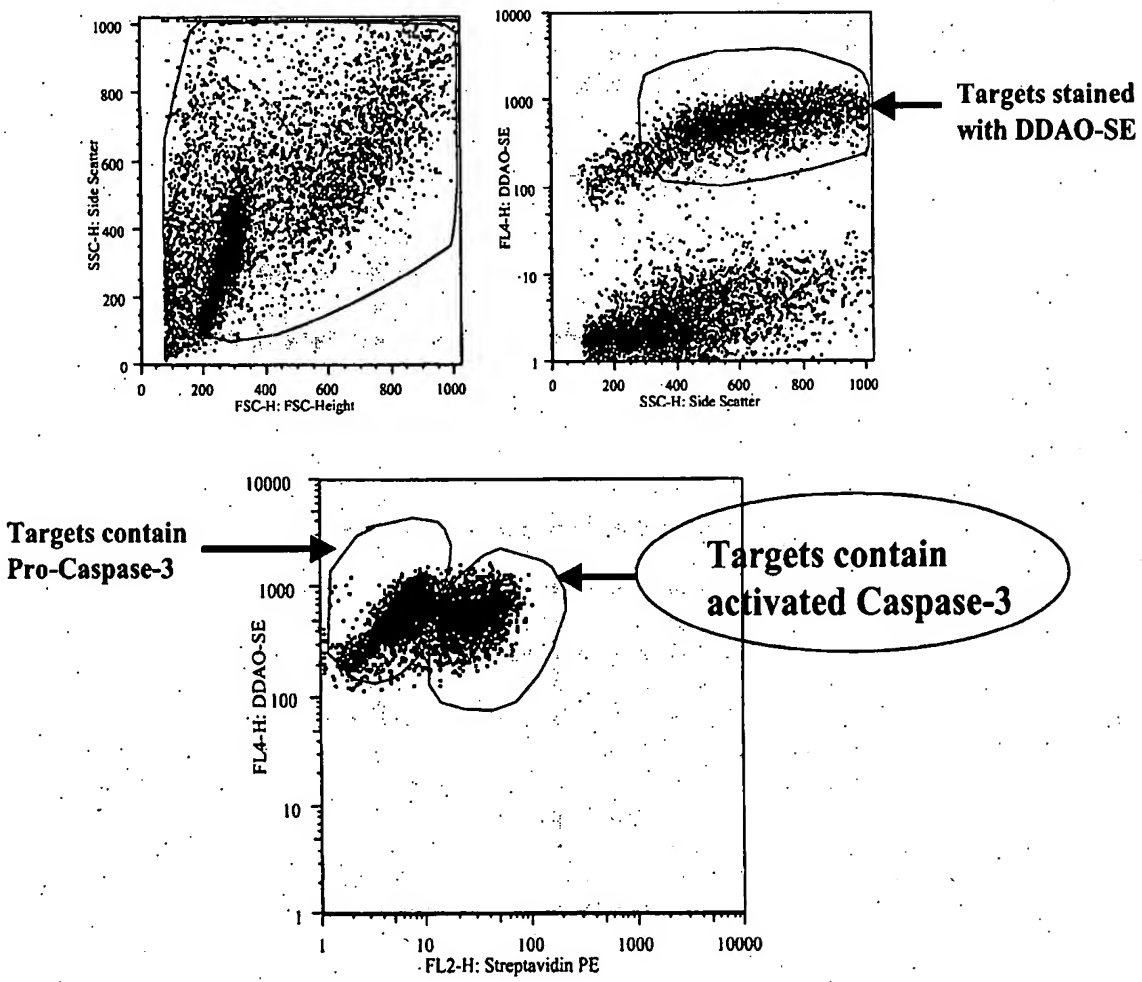

FIGURE 3
Targets: P815

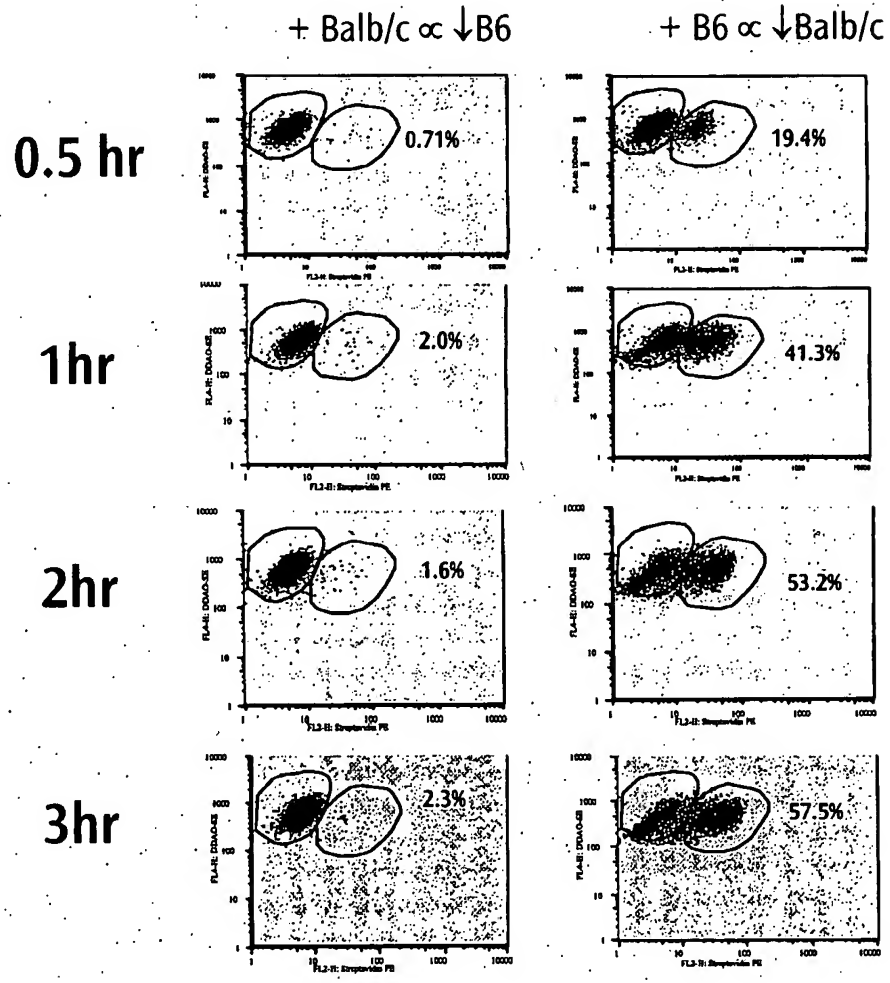

FIGURE 4

Mouse MLR CTL Activity

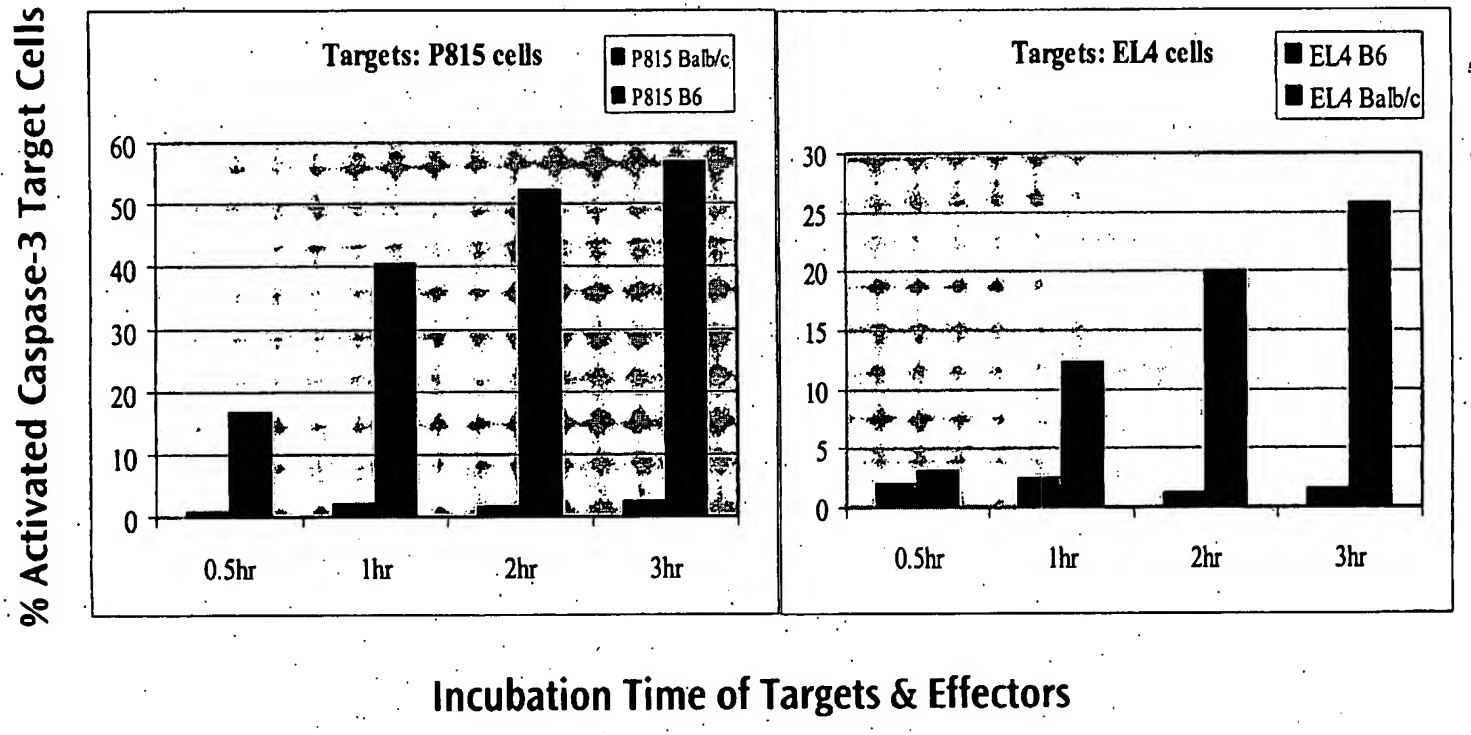

FIGURE 5

Antigen Specific Mouse CTL Assay

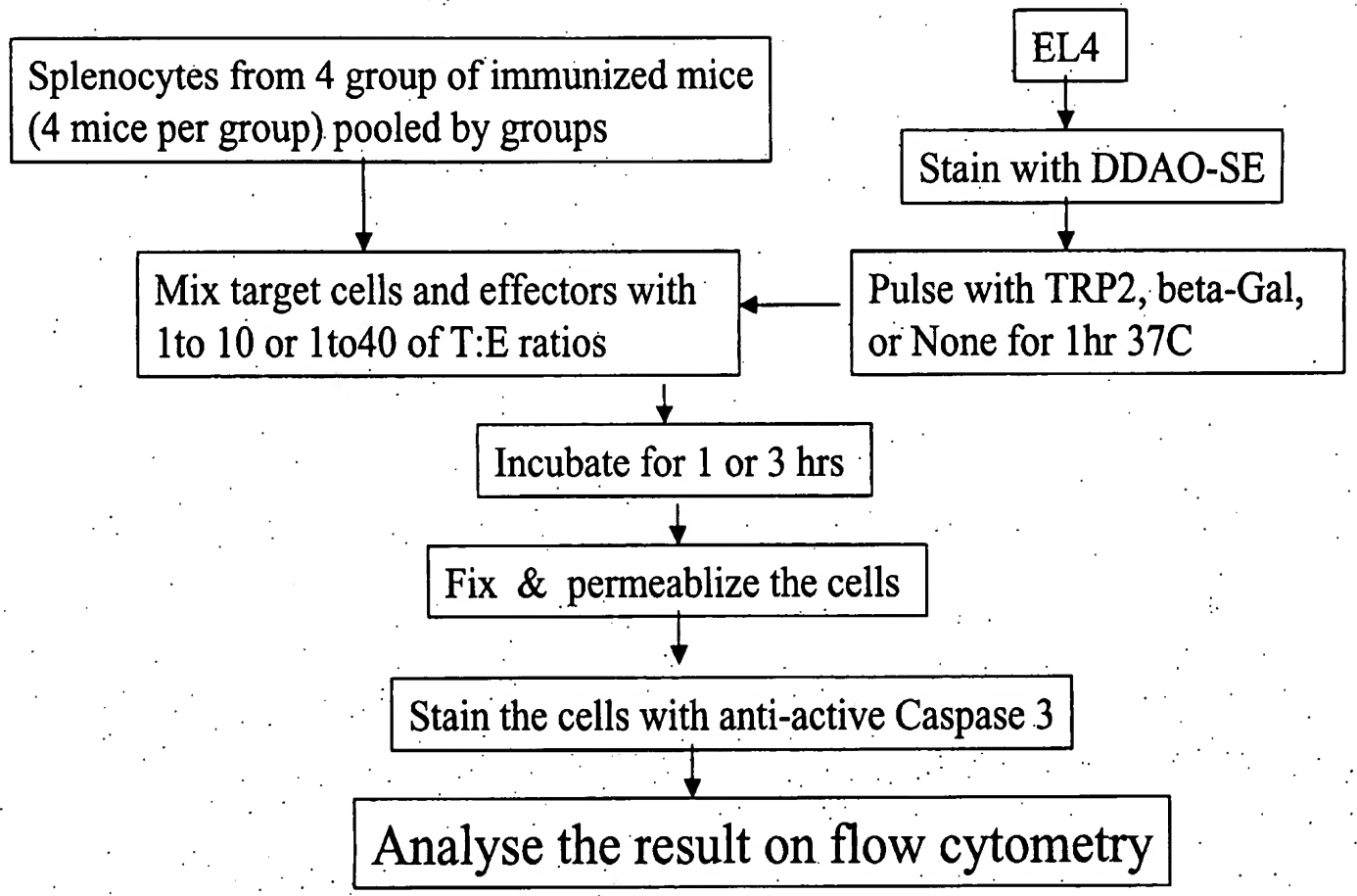

FIGURE 6
STUDY DS-27
Assessment of immune response to TRP2 peptide linked to hPER1 PTD sequence

Group	#of mice	Procedure
A	4	Day 0: s.c. injection of 50nmoles TRP2 + 50nmoles of CLP243 in 4 mice Day 21: Boost Day 42: harvest 4 spleens
B	4	Day 0: s.c. injection of 50nmoles hPER1- FVYVW-TRP2 + 50nmoles of CLP243 in 4 mice Day 21: Boost Day 42: harvest 4 spleens
C	4	Day 0: s.c. injection of 50nmoles hPER1-L-TRP2 + 50nmoles of CLP243 in 4 mice Day 21: Boost Day 42: Harvest 4 spleens
D	4	Day 0: s.c. injection of 50nmoles hPER1-TRP2 + 50nmoles of CLP243 in 4 mice Day 21: Boost Day 42: Harvest 4 spleens
E	4	Day 0: s.c. injection of 50nmoles TRP2 + 50nmoles of CLP243 in IFA in 4 mice Day 21: Boost Day 42: Harvest 4 spleens

FIGURE 7

Peptide-specific Mouse CTL Assay

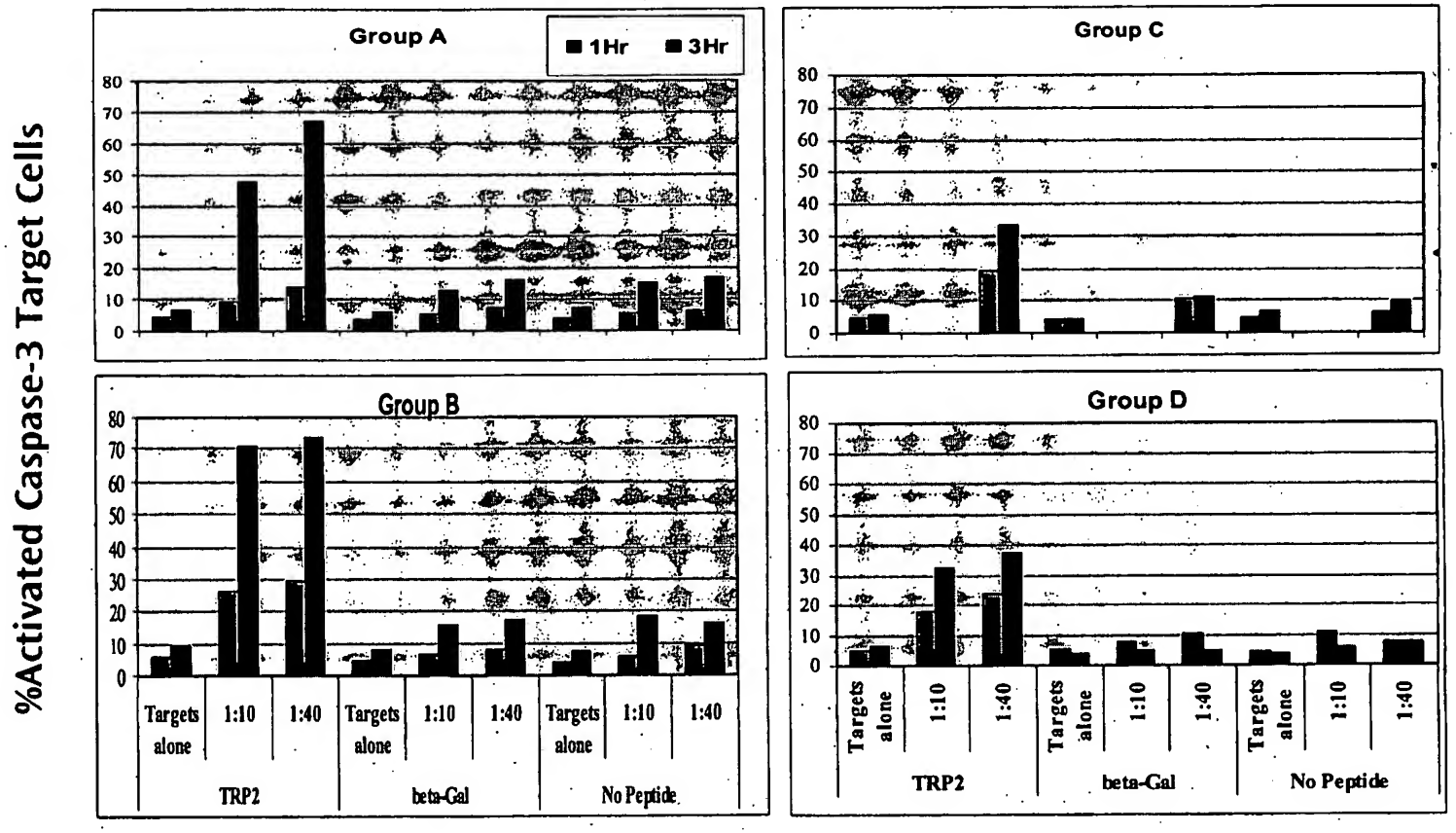

FIGURE 8

Human T cell Activation CTL Assay

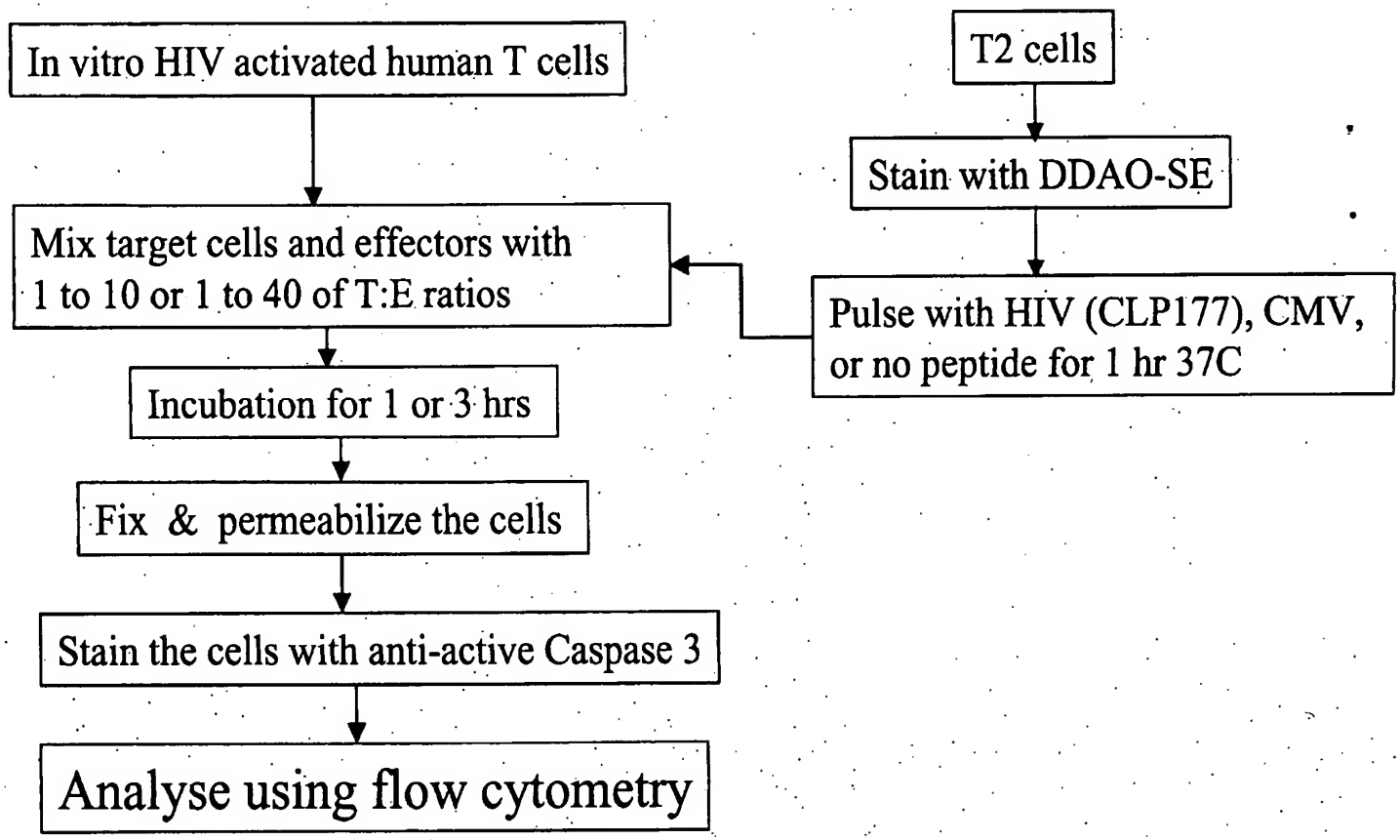

FIGURE 9
Human CTL Assay Results

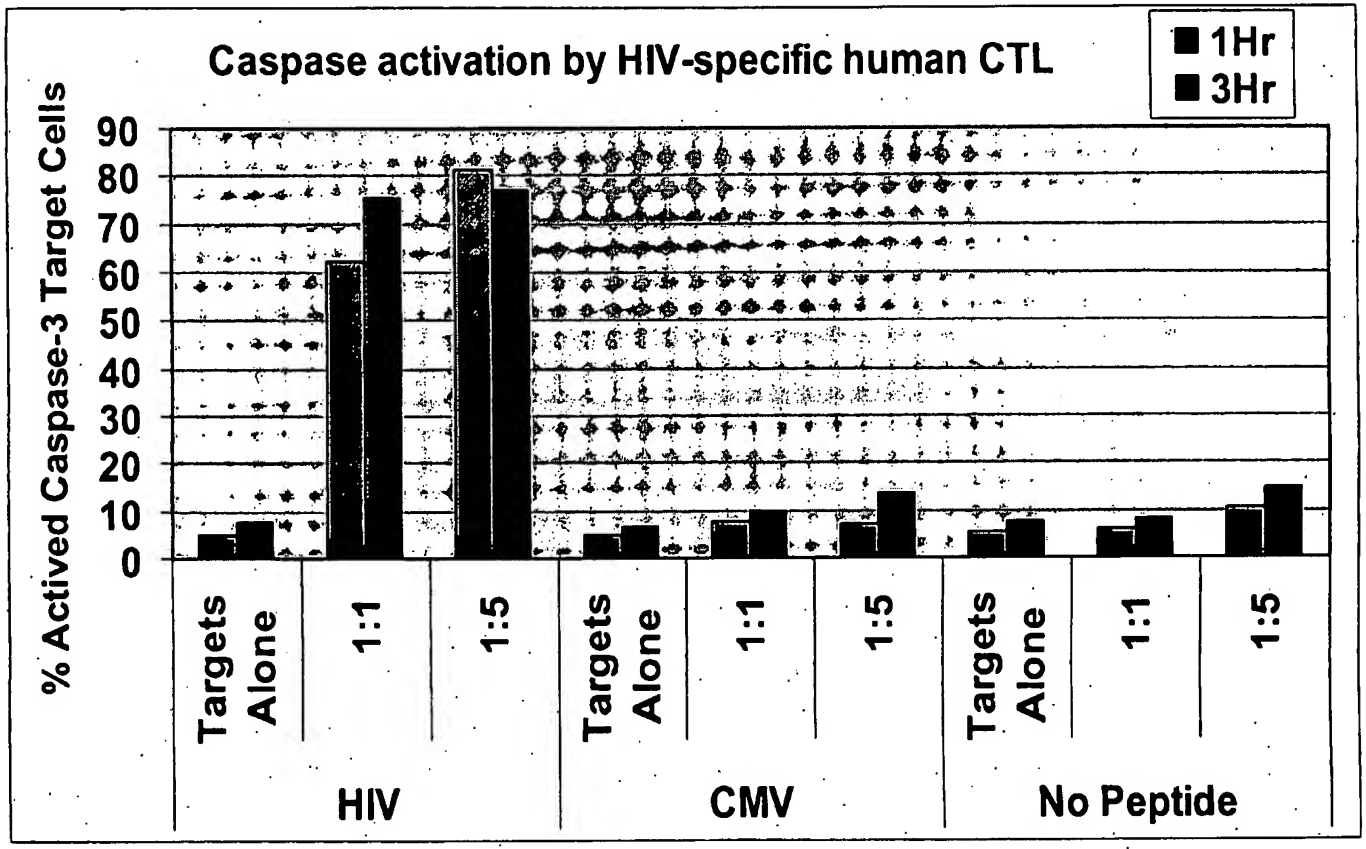

FIGURE 10

Dilution of Effectors with Naïve Cells

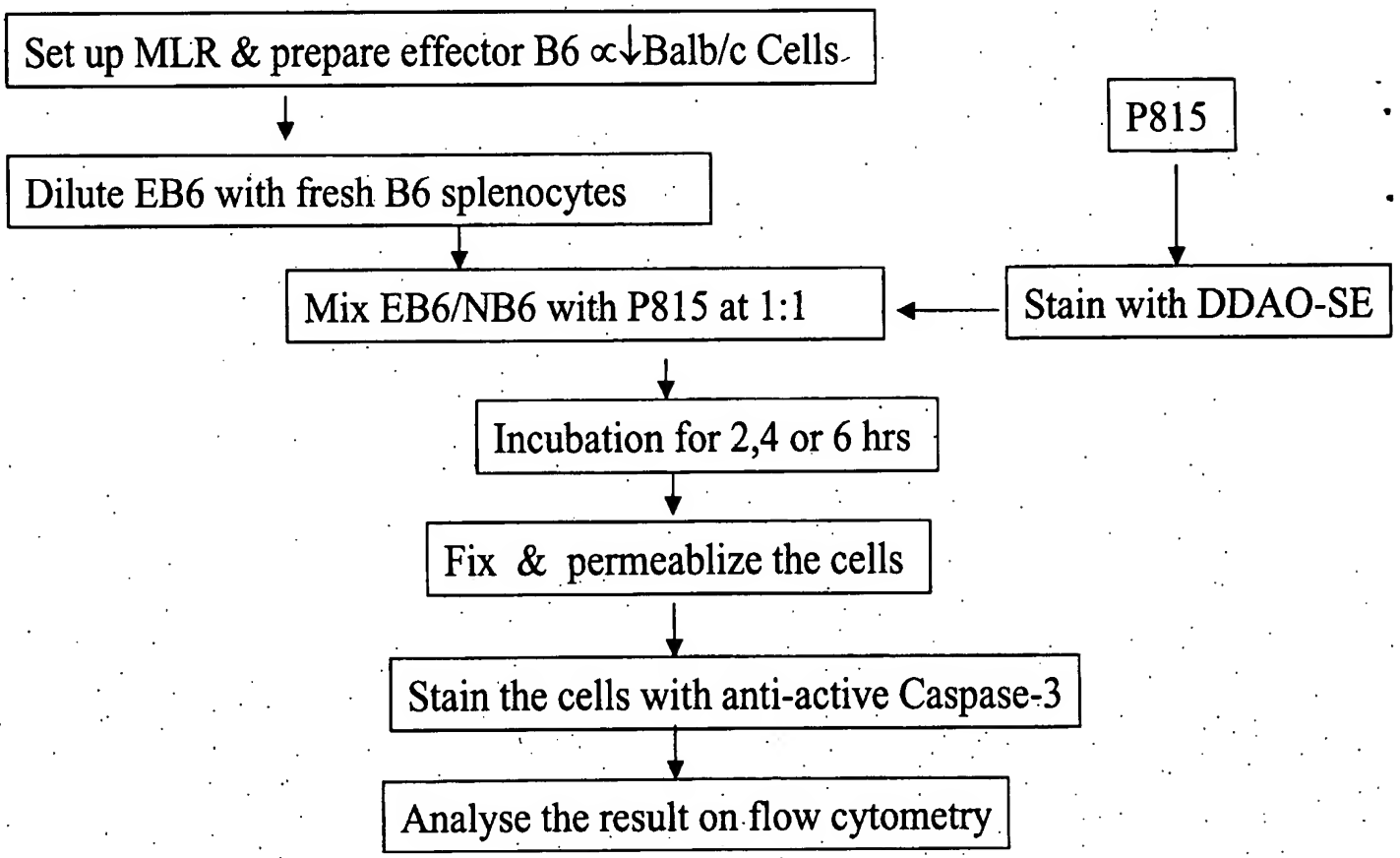

FIGURE 11
Dilution of Effectors with Naïve Splenocytes

Effector B6: Naïve B6	# Effector B6 /well	# Naïve B6/well	P815: Effector B6
Effector B6 only	150,000	0	1:1
1:1	75,000	75,000	2:1
1:3	37,500	112,500	4:1
1:7	18,750	131250	8:1
1:15	9,375	140,625	16:1
1:31	4,688	145,312	32:1
1:63	2,344	147,656	64:1
1:99	1,500	148500	100:1
1:199	750	149250	200:1
Naïve B6 only	0	150,000	1:0

FIGURE 12

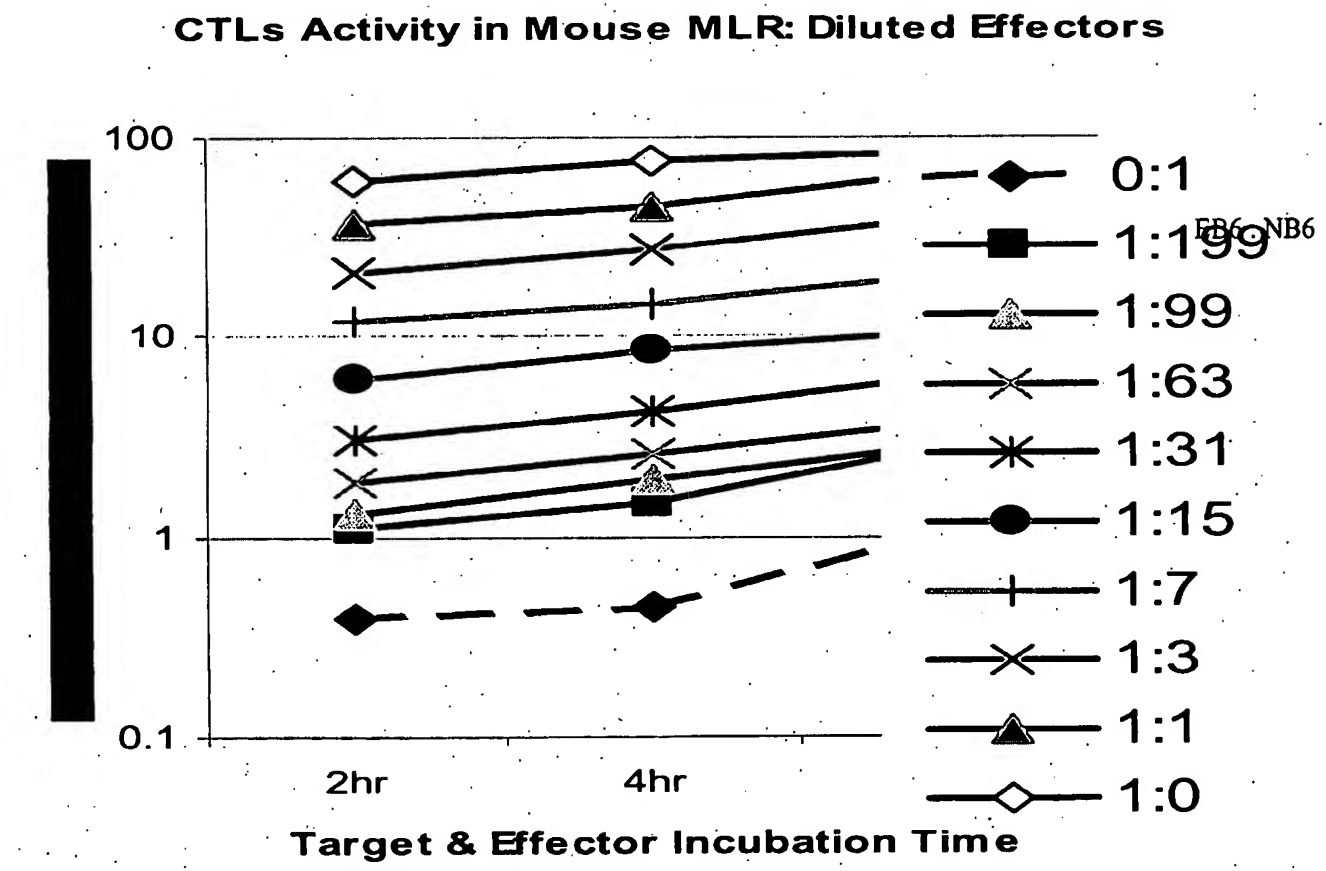
CTLs Activity in Mouse MLR: Diluted Effectors
100
10
1
0.1
2hr
4hr
Target & Effector Incubation Time
0:1
1:199
1:99
1:63
1:31
1:15
1:7
1:3
1:1
1:0

FIGURE 13
Sensitivity of Caspase-3 Assay in Effector Dilution

B6 effector: B6 naive	Effector B6 in Total Cells (%)	Frequency of Caspase-3 Positive Targets detected in total cells (%)		
		2hr	4hr	6hr
Effector B6 alone	50	26.90	33.33	41.88
1:1	25	17.58	21.57	33.58
1:3	12.5	10.26	13.81	20.49
1:7	6.25	5.99	7.61	11.05
1:15	3.13	3.03	4.41	5.75
1:31	1.56	1.56	2.27	3.48
1:63	0.78	0.98	1.35	2.09
1:99	0.5	0.68	1.03	1.60
1:199	0.25	0.58	0.79	1.56
Naïve B6 alone	0	0.16	0.19	0.45

FIGURE 14
2hr Incubation

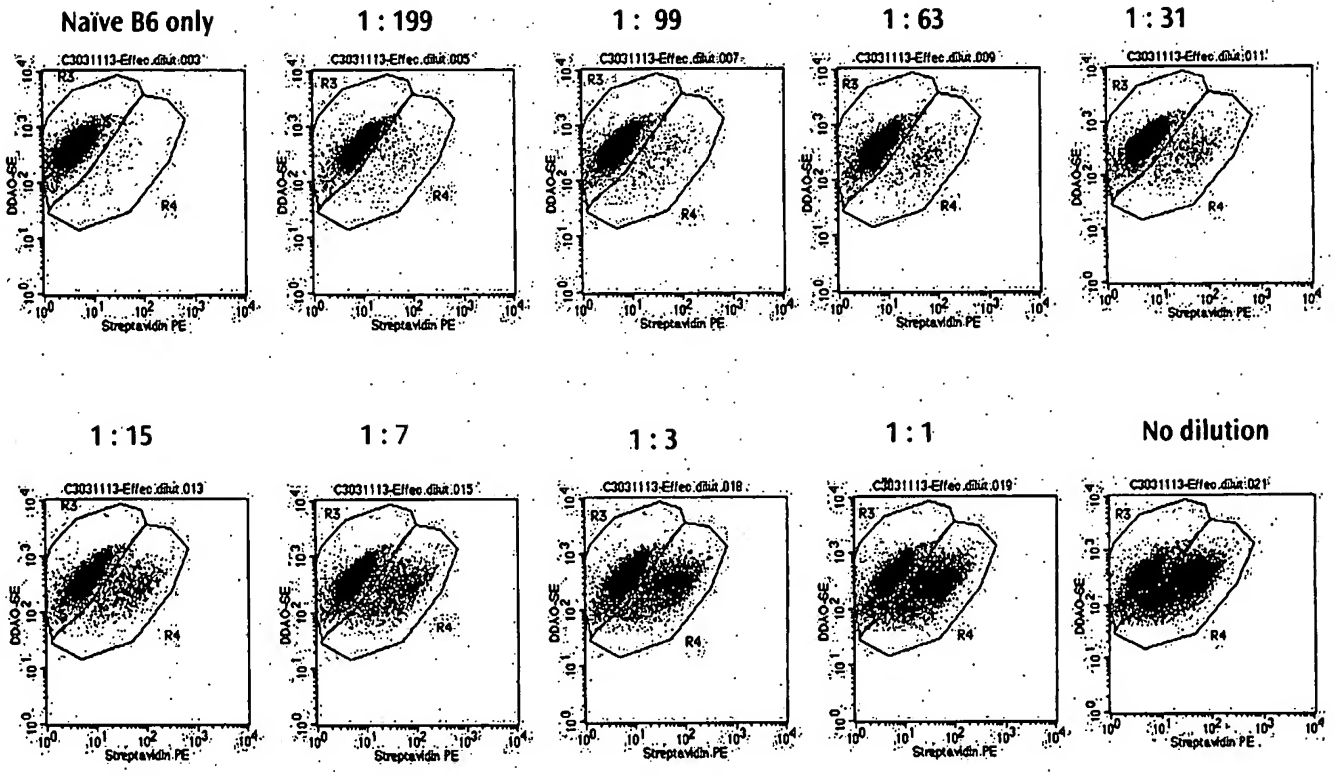

FIGURE 15
4hr Incubation

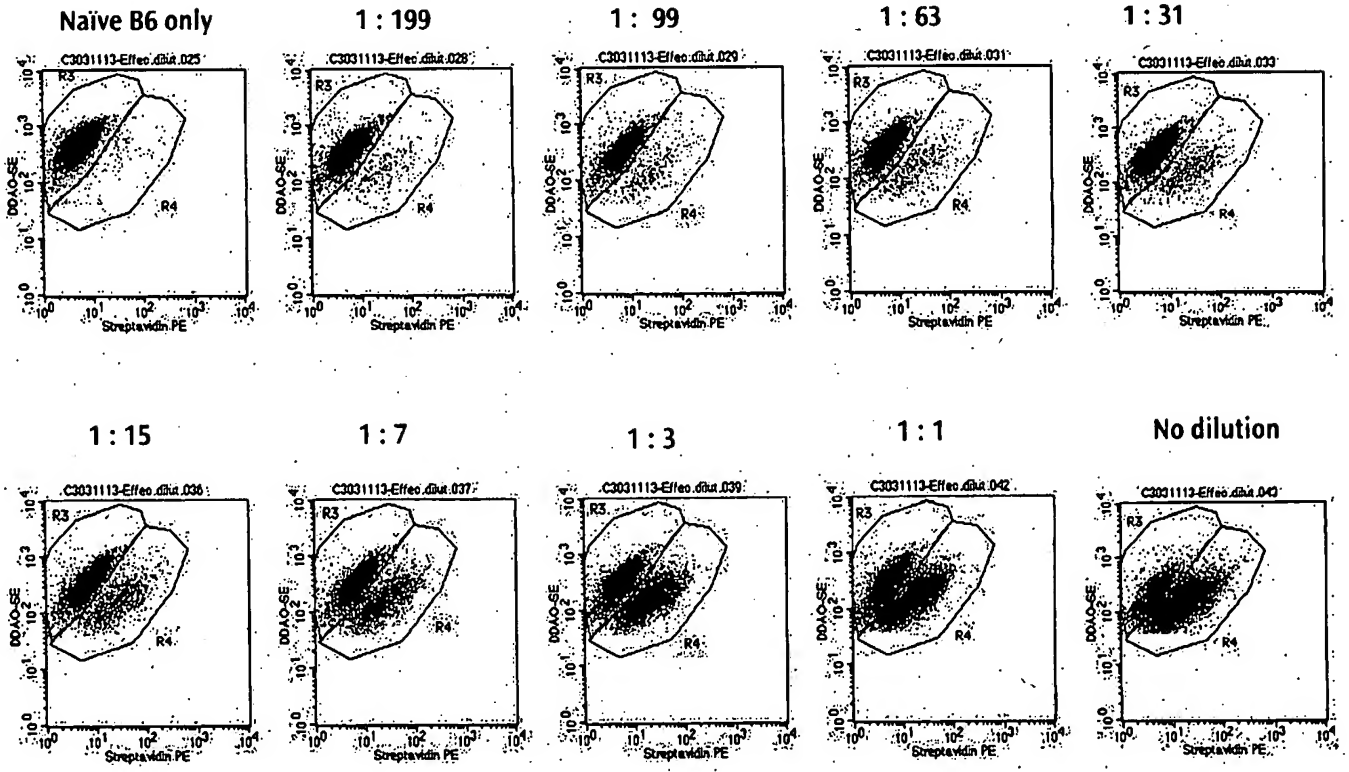

FIGURE 16
6hr Incubation